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Phosphatidylinositol 3-Kinase Activity Negatively Regulates Stability of Cyclooxygenase 2 mRNA*

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Human alveolar macrophages have both lipopolysaccharide (LPS)-induced and constitutive phosphatidylinositol 3-kinase (PI3K) activity. We observed that blocking PI3K activity increased release of prostaglandin E2 after LPS exposure, and increasing PI3K activity (interleukin-13) decreased release of prostaglandin E2 after LPS exposure. This was not because of an effect of PI3K on phospholipase 2 activity. PI3K inhibition resulted in an increase in cyclooxygenase 2 (COX2) protein, mRNA, and mRNA stability. PI3K negatively regulated activation of the p38 pathway (p38, MKK3/6, and MAPKAP2), and an active p38 was necessary for COX2 production. The data suggest that PI3K inhibition of p38 modulates COX2 expression via destabilization of LPS-induced COX2 mRNA.

Alveolar macrophages are major effector cells of the innate immune system. They play a central role in the response to Gram-negative bacteria in the lung. Endotoxin (LPS)[1] is the principle activating component of the Gram-negative cell wall and is a major activator of these macrophages. After initial exposure to LPS, expression of prostaglandin endoperoxide H synthase 2 (COX2) and production of prostaglandin E2 (PGE2) appears at 12 to 24 h after stimulation (1). Cyclooxygenases (COXs) catalyze the conversion of arachidonic acid and O_2 to PGH2. It is the rate-limiting step in the metabolism of arachidonic acid to prostanoid products. Arachidonic acid is a 20-carbon unsaturated fatty acid that is hydrolyzed from membrane-bound phospholipids by the actions of phospholipases (PLA) (secretory PLA2 and cytosolic PLA2). Both COX1 and -2 catalyze the same step in the arachidonic acid pathway (a cyclooxygenase reaction in which arachidonic acid is converted to PGG2 and a peroxidase reaction in which PGG2 is reduced to PGH2) (2, 3). COX1 is a constitutively present enzyme, and its products are thought to be important in gastric and renal homeostasis (4, 5). It is the only cyclooxygenase present in platelets and has been linked to platelet production of thromboxane A2. In contrast, COX2 is induced by inflammatory mediators and has been linked to inflammation, fever, pain, and a number of cancers (6). The preferred prostanoid products of COX2 are prostacyclin and PGE2 (5). We have shown that in alveolar macrophages LPS induces COX2 expression and PGE2 release (1, 7–9).

Phosphatidylinositol 3-kinase (PI3K) is a heterodimeric dual function lipid and protein kinase that has been linked to cell survival, transcription factor activation, and multiple signaling pathways (10–12). Class 1A PI3Ks (found in alveolar macrophages) consist of a p85-kDa subunit protein (α and β) and a p110-kDa catalytic subunit (α, β, and δ) or a p101-kDa regulatory unit and a p110-kDa catalytic unit (γ). The p85 regulatory unit is activated via interaction of the SH2 domain with Y*XX*M motifs of multiple receptors. The p101 regulatory unit is activated by $\gamma\beta$ subunits of G proteins downstream of G protein-coupled receptors (10). Once activated PI3K catalyzes the transfer of ATP to the D-3 position of the inositol ring of membrane-localized phosphoinositides. This results in the production of a number of bioactive lipid species including PI_3P, $PI_{3,4}P$, and $PI_{3,4,5}P$. Both $PI_{3,4}P$ and $PI_{3,4,5}P$ are absent in most unstimulated cells and increase dramatically following PI3K activation. The presence of $PI_{3,4,5}P$ results in the membrane recruitment of proteins containing pleckstrin homology domains. This includes PI3K-dependent kinase (PDK-1), which phosphorylates a number of biologically important substrates (Akt, protein kinase A, and multiple protein kinase C isoforms) (13). We have shown previously that LPS activates Akt via activation of PI3K in alveolar macrophages (14–16). Activation of Akt is linked to NFκB translocation and transactivation, endothelial nitric oxide synthase activation, and inhibition of a number of substrates positively involved in apoptosis. The apoptosis-related factors that are inhibited by Akt include glycogen synthase kinase 3, forkhead transcription factors, Bad, and caspase 9 (17). Glycogen synthase kinase 3 inhibition results in increased signaling from a number of transcription factors, β catenin, nuclear factor of activated T-cells, CCAAT/enhancer binding protein, GATA 4, and some of the activator protein 1 proteins (18). Activation of PI3K is therefore linked to multiple biological effects. One possible role of PI3K activity is as a modulator of MAP kinase signaling. Akt, in some conditions, has been shown to negatively regulate c-Raf (part of the ERK

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[1] The abbreviations used are: LPS, lipopolysaccharide; COX, cyclooxygenase; PG, prostaglandin; PLA, phospholipase; PI3K, phosphatidylinositol 3-kinase; NFκB, nuclear factor κB; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH_2-terminal kinase; ELISA, enzyme-linked immunosorbent assay; C_t, threshold cycles; MKK3/6, mitogen-activated protein kinase kinase 3/6; MAPKAP2, mitogen-activated protein kinase activated protein kinase-2; HPRT, hypoxanthine phosphorilosyltransferase; PI, phosphatidylinositol; P, phosphate.

pathway in some cells) (19), p38 (20), and stress-activated protein kinase kinase (upstream of JNK) (21).

The MAP kinases are a family of evolutionarily conserved enzymes that connect cell surface receptors to regulatory targets that include both cytoplasmic and nuclear proteins. The three major MAP kinase families are the ERK (1 and 2), p38 (α, β, γ, and δ), and the JNK (1, 2, and 3) (22). LPS exposure leads to the activation of all three MAP kinase pathways (ERK, p38, and JNK) (23, 24), and activation of ERK and p38 has been linked to COX2 expression (25, 26). p38 phosphorylates both cytoplasmic and nuclear substrates. Non-transcription factor effects of p38 include increasing mRNA stability and phosphorylation of basal transcription complex components (27, 28). p38 activity has been linked to regulation of cytokine mRNA stability via its inhibitory actions on the mRNA destabilizing protein tristetraprolin (29). More specifically, p38 activation has been linked to COX2 mRNA stability (27, 30, 31).

In these studies, we found that inhibition of the PI3K pathway increased LPS-induced COX2 and PGE2. Lack of PI3K activity increased the stability of COX2 mRNA, and this increased stability correlated with increased PGE2 release. We found PI3K activity to be correlated inversely with LPS-induced p38 activity. Inhibition of PI3K resulted in increased p38 activity. These studies suggest that constitutive and LPS-induced PI3K activity in alveolar macrophages delays and decreases the production of COX2 and release of PGE2.

EXPERIMENTAL PROCEDURES

Materials—Chemicals were obtained from Sigma and Calbiochem. Protease inhibitors were obtained from Roche Molecular Biochemicals. LPS was obtained from LIST Biologicals, Campbell, CA, and LY294002 was from Calbiochem. IL-13 was from R&D Systems, Minneapolis, MN. Nitrocellulose and ECL Plus were obtained from Amersham Biosciences. Antibodies were obtained from various sources; antibodies to ERK, p38, MKK3, and MAPKAP2 were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Antibody to COX1 and COX2 was obtained from Cayman Chemicals, Ann Arbor, MI. Phosphorylation-specific antibodies to ERK and p38 were obtained from Sigma, and all other phosphorylation-specific antibodies were from Cell Signaling, Beverly, MA.

Isolation of Human Alveolar Macrophages—Alveolar macrophages were obtained from normal non-smoking volunteers, as described previously (32). Briefly, normal volunteers with a lifetime non-smoking history, no acute or chronic illness, and no current medications underwent bronchoalveolar lavage. The cell pellet was washed twice in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} and suspended in complete medium, RPMI tissue culture medium (Invitrogen) with 100 ng/ml lipopolysaccharide-binding protein (a gift from Peter Tobias, Scripps Research Institute, La Jolla, CA) and added to gentamycin (80 μg/ml). Differential cell counts were determined using a Wright-Giemsa-stained cytocentrifuge preparation. All cell preparations had between 90–100% alveolar macrophages. This study was approved by the Committee for Investigations Involving Human Subjects at the University of Iowa.

Cell Culture—Alveolar macrophages were cultured in complete medium at 1–5 $\times$ 10^6 cells per ml depending on the experiment. Most cultures were done in 1.8-ml microfuge tubes at 37 °C, 5% CO_2. Inhibitors (LY294002 at 10 μM or SB202190 at 10 μM) were added 30 min before LPS (100 ng/ml).

Isolation of Whole Cell Extracts—Whole cell protein was obtained by lysing the cells on ice for 20 min in 500 μl of lysis buffer (0.05 M Tris, pH 7.4, 0.15 M NaCl, 1% Nonidet P-40, 1 protease minitab (Roche Molecular Biochemicals)/10 ml, and 1$\times$ phosphatase inhibitor mixture (catalog number 524625; Calbiochem). The lysates were then sonicated for 20 s and incubated at 4 °C for 30 min, and the insoluble fraction was removed by centrifugation at 15,000 $\times$ *g* for 10 min. Protein determinations were made using a protein measurement kit from Bio-Rad (Hercules, CA).

Western Analysis—Western analysis for the presence of particular proteins or for phosphorylated forms of proteins was performed as described previously (33). 50 μg of protein was mixed 1:1 with 2$\times$ sample buffer (20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.05% bromphenol blue, and 1.25 M Tris, pH 6.8) and loaded onto a 10% SDS-PAGE gel and run at 30 mA for 3 h. Cell proteins were transferred to nitrocellulose with a Bio-Rad semi-dry transfer system, according to the manufacturer's instructions. Equal loading of the protein groups on the blots was evaluated using Ponceau S, a staining solution designed for staining proteins on nitrocellulose membranes. The nitrocellulose was then blocked with 5% milk in TTBS (Tris-buffered saline with 0.1% Tween 20) for 1 h, washed, and then incubated with the primary antibody at dilutions of 1:500 to 1:2000 overnight. The blots were washed four times with TTBS and incubated for 1 h with horseradish-peroxidase conjugated anti-IgG antibody (1:5000 to 1:20,000). Immunoreactive bands were developed using a chemiluminescent substrate, ECL Plus (Amersham Biosciences). An autoradiograph was obtained, with exposure times of 10 s to 2 min.

PGE2 Release—Alveolar macrophages were cultured in standard medium for 24 or 48 h with and without LPS (100 ng/ml) or LY294002 or both. In some experiments, IL-13 (10 ng/ml) was added 30 min before the LPS. After the culture period, the supernatants were harvested and stored at −70° until they were assayed. The amount of PGE2 in the supernatant was measured by ELISA. (Amersham Biosciences).

Isolation of RNA—Total RNA was isolated using the Absolutely RNA RT-PCR miniprep kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. RNA was quantitated using a RiboGreen kit (Molecular Probes, Eugene, OR). RNA samples were stored at −70 °C.

Real-time RT-PCR Detection of COX2 mRNA—1 μg of total RNA was reverse-transcribed to cDNA using a RETROscript RT-PCR kit (Ambion, Austin, TX). The resulting cDNA was subjected to PCR in a Bio-Rad iCycler iQ system as follows: in a 0.2-ml PCR tube (Bio-Rad), 2 μl of cDNA was added to 48 μl of PCR reaction mixture containing 2 mM each dNTP (Invitrogen), 3.0 mM $MgCl_2$ (Invitrogen), 1:15,000 SYBR Green I DNA dye (Molecular Probes, Eugene, OR), 0.2 μM of each sense and antisense primer (IDT, Coralville, IA), and 2.5 units of Platinum *Taq*DNA (Invitrogen). Amplification and data collection was performed as described previously (34). Primers for human COX2 and HPRT genes are as follows (5′ to 3′): COX2 sense, TCAGCCATACAGCAAATCCTT; COX2 antisense, GTGCACTGTGTTTGGAGTGG; HPRT sense, CCTCATGGACTGATTATGGAC; HPRT antisense, CAGATTCAACTTGCGCTCATC.

Quantitation of COX2 mRNA—Relative quantitative gene expression was calculated as follows. For each sample assayed, the threshold cycles (C_t) for reactions amplifying COX2 and HPRT were determined. The COX2 C_t for each sample was corrected by subtracting the C_t for HPRT (ΔC_t). Untreated controls were chosen as the reference samples, and the ΔC_t for all LPS-treated experimental samples were subtracted by the ΔC_t for the controls samples ($\Delta\Delta C_t$). Finally, LPS-treated COX2 mRNA abundance, relative to control COX2 mRNA abundance, was calculated by the formula $2 - (\Delta\Delta C_t)$. Validity of this approach was confirmed by using serial 10-fold dilutions of template containing COX2 and HPRT genes. Using this set of template mixtures, the amplification efficiencies for COX2 and HPRT amplimers were found to be identical.

Statistical Analysis—Statistical analysis was performed on densitometry data, ELISA results, and real-time PCR data. Significance was determined by Student's *t* test.

RESULTS

PGE2 Production by LPS-treated Alveolar Macrophages Is Modulated by PI3K Activity—For these studies, some of the cells were treated with LPS and/or the PI3K inhibitor, LY294002. LY294002 is a competitive inhibitor that binds to the ATP-binding site of PI3K (35, 36). We have shown that in alveolar macrophages, LY294002 blocks PI3K activation and phosphorylation of Akt (15, 16). After culture for various periods of time, supernatants were harvested, and PGE2 was measured. LPS stimulation of alveolar macrophages resulted in increased amounts of PGE2. Significant levels of PGE2 first appear around 24 h and continue expanding out to 48 h (Fig. 1*A*). The amounts of PGE2 were increased by inhibition of PI3K activity. At 24 h the increase with PI3K inhibition was 3- to 4-fold (Fig. 1*B*). At 48 h the increase was 8- to 10-fold. Because we found a significant difference in PGE2 at 24 h, and a 24-h culture period allowed us to avoid the significant viability differences in alveolar macrophages found at 48 h, the remaining experiments were done at 24 h. These data demonstrate that inhibition of the PI3K pathway increases the release of PGE2 after exposure to LPS significantly. The Th2 cytokine,

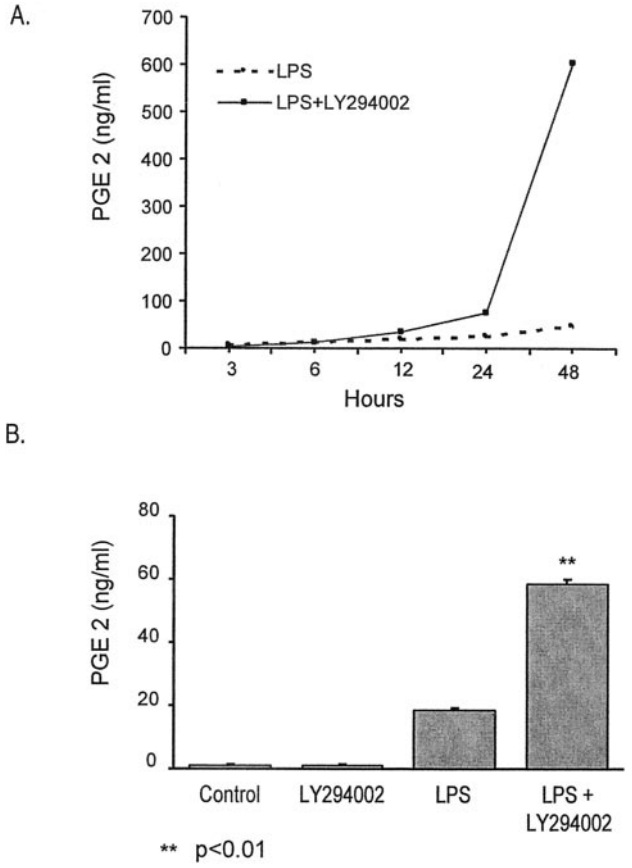

FIG. 1. **Inhibition of PI3K increases LPS-induced PGE2.** *A*, alveolar macrophages were cultured with and without LPS (100 ng/ml) and a PI3K inhibitor, LY294002 (10 μm). After various time points (3–6 h) in culture, the supernatant was removed, and PGE2 levels were determined by ELISA. *B*, the same experiment was performed on cells from multiple donors (6), and PGE2 levels were determined by ELISA. Statistical analysis was done using an unpaired Student's *t* test. **, $p < 0.01$.

IL-13, is a strong activator of the PI3K pathway (37, 38). Therefore, we treated the cells with IL-13 (10 ng/ml) and LPS and evaluated release of PGE2. First we evaluated the effect of IL-13 on Akt activation in alveolar macrophages. We found that, consistent with the literature, IL-13 activated Akt (Fig. 2*A*). The administration of IL-13 caused a decrease in LPS-induced PGE2 release (~70%) (Fig. 2*B*). These two pieces of data (LY294002-dependent increases in PGE2 and IL-13-dependent decreases in PGE2) suggest that the PI3K pathway regulates LPS-induced PGE2 production negatively.

PI3K Is Constitutively Active in Alveolar Macrophages—In alveolar macrophages, Akt activation depends on PI3K activity and can be used as an indicator of active PI3K (15). Akt activation occurs through phosphorylation of threonine 308 in the activation loop, followed by autophosphorylation of serine 473 in the carboxy-terminal region (39). We evaluated phosphorylation of serine 473 as a marker of both PI3K and Akt activity. To determine whether there is high baseline PI3K activity in alveolar macrophages, we obtained cells from four normal volunteers, cultured them for 30 min with LY294002, and obtained whole cell lysates. Examining newly isolated alveolar macrophages, we found significant amounts of phosphorylated Akt at baseline that was decreased by LY294002 incubation (Fig. 3). These data, combined with our earlier studies showing increased activation of PI3K after LPS, suggest that alveolar macrophages have a mechanism (PI3K activity) that dampens production of PGE2.

PI3K Activity Decreases at Later Time Points after LPS Exposure—Having found significant amounts of baseline and LPS-induced PI3K activity at early time points (5 min to 3 h) (see Fig. 3 and earlier studies (15, 16)), we next evaluated long term PI3K activation after exposure to LPS. We found that there was a time-dependent decrease in Akt activation (Fig. 4). Akt activation was decreased to below baseline levels by 4 to 6 h after LPS and continued to decrease further out to 24 h. These data suggest that the inhibitory effect of PI3K on PGE2 decreases as cells begin to release PGE2.

PI3K Inhibition Does Not Increase COX1 or PLA2 Activity—Inflammation-induced PGE2 production is regulated by the availability of arachidonic acid and by the production of COX2. Arachidonic acid is cleaved from phospholipids by PLA2. This provides a substrate for peroxidase, cyclooxygenase, and PGE synthase activities that lead to the generation of PGE2. The increased PGE2 seen with PI3K inhibition could be because of

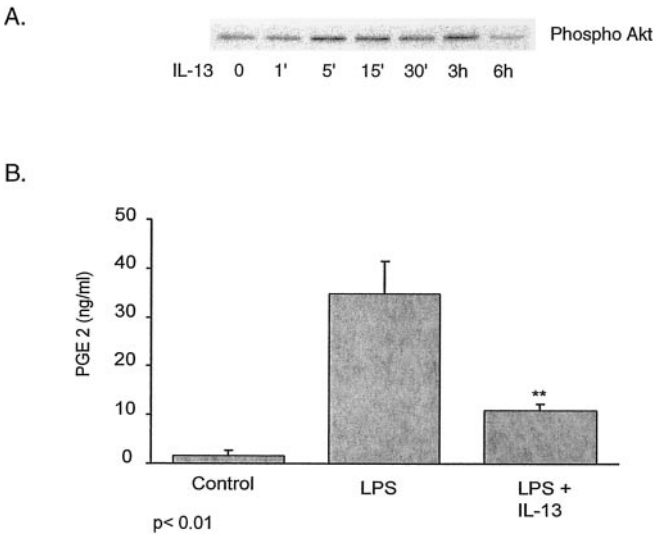

FIG. 2. **IL-13 activates Akt and inhibits LPS-induced PGE2.** *A*, alveolar macrophages were cultured with IL-13 (10 ng/ml) for various times (1 min to 6 h), whole cell lysates were obtained, and Western analysis was performed for Akt phosphorylation (serine 473) as described under "Experimental Procedures." *B*, alveolar macrophages were cultured with and without LPS (100 ng/ml) and IL-13 (10 ng/ml) for 24 h. The supernatant was removed, and PGE2 levels were determined by ELISA. The data represent three separate experiments. **, $p < 0.01$.

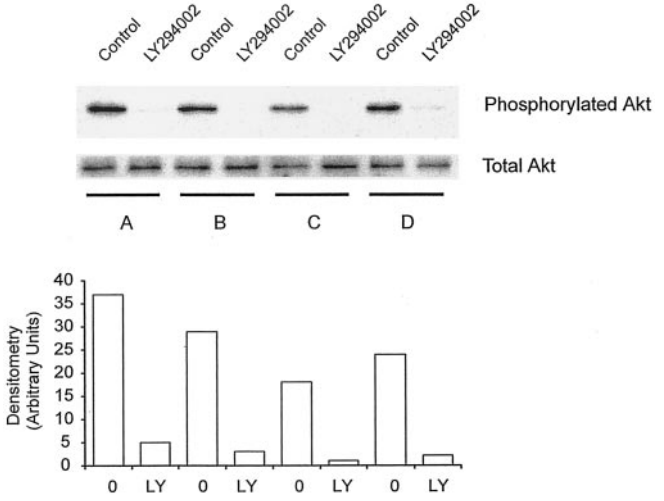

FIG. 3. **Alveolar macrophages have constitutive PI3K activity.** Alveolar macrophages were treated with or without LY294002 (10 μM) for 30 min immediately after isolation. Whole cell protein was obtained, and Western analysis was performed for Akt phosphorylation (serine 473). *A–D* designate four different bronchoalveolar lavage volunteers.

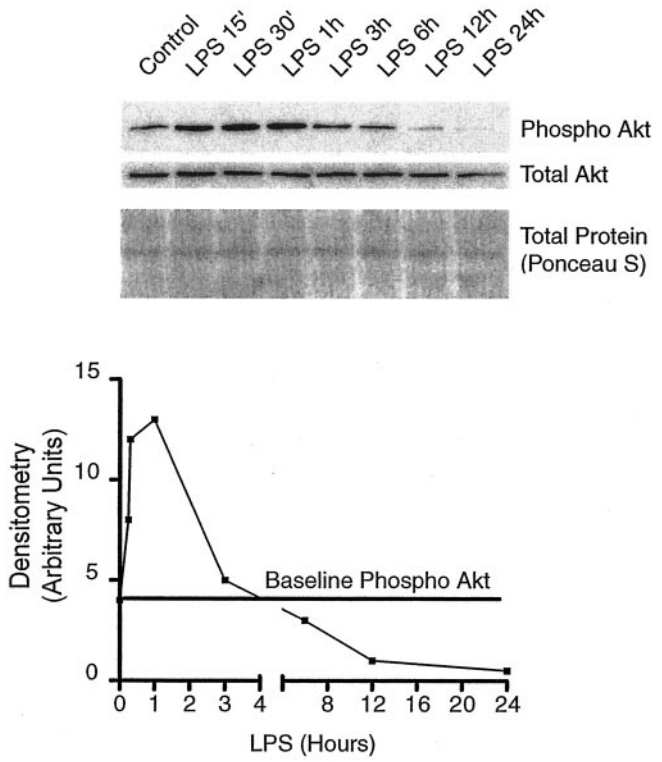

FIG. 4. **LPS increases and then decreases Akt activation.** Alveolar macrophages were cultured with LPS (100 ng/ml) for various times (15 min to 24 h). Whole cell proteins were obtained, and Western analysis was performed for phosphorylated Akt (serine 473). This is a representative of four different experiments.

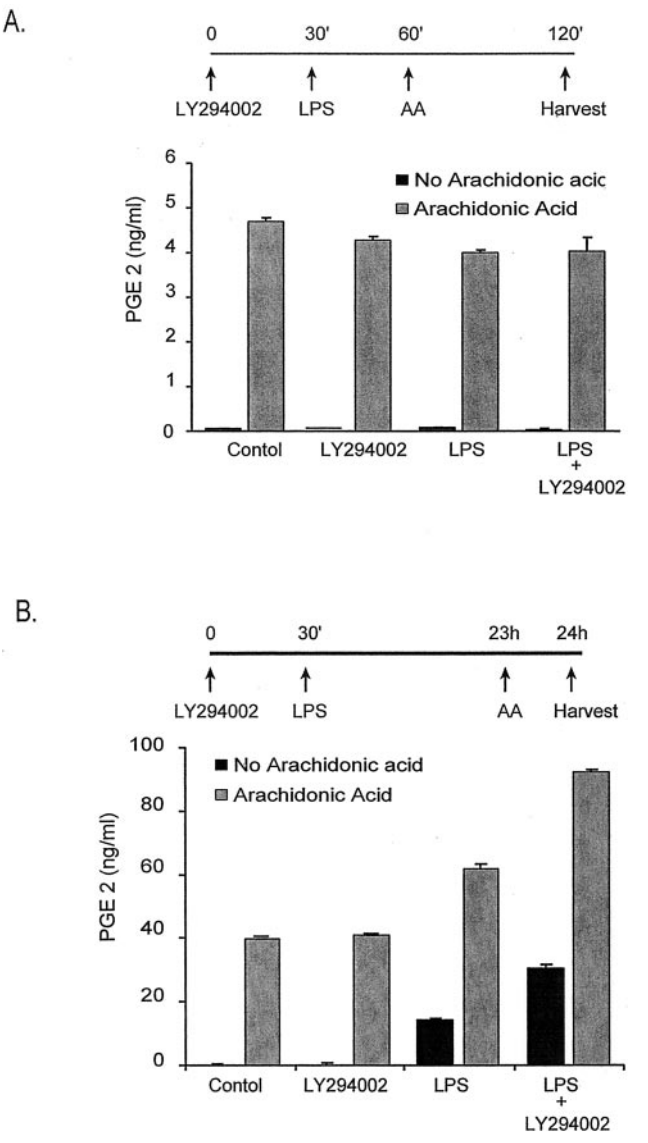

FIG. 5. **Inhibition of PI3K does not alter COX1 or PLA2 activity.** *A*, alveolar macrophages were cultured for 3 h with and without LPS (100 ng/ml) and LY294002 (10 μm). After 30 min, arachidonic acid (100 μM) was added to provide a substrate for the endogenous COX1. At 3 h supernatants were harvested, and PGE2 levels were determined by ELISA. *B*, alveolar macrophages were cultured for 24 h with and without LPS (100 ng/ml) and LY294002 (10 μm). After 23 h, arachidonic acid (100 μM) was added to bypass an effect of LY294002 on PLA2 activity. At 24 h supernatants were harvested, and PGE2 levels were determined by ELISA. The data represent three separate experiments. **, $p < 0.01$.

either increased production of COX2 or increased PLA2 activity. To determine whether the increased PGE2 was because of increased PLA2 activity or activation of the endogenous COX1, we bypassed PLA2 by providing exogenous arachidonic acid. The arachidonic acid was dried, resuspended in media, and added directly to the cultures. Previous studies have shown that arachidonic acid is taken up very quickly (5 min) by macrophages when added in this manner (40). Fig. 5*A* shows that when the cell is provided with free arachidonic acid at early time points there is no difference in the amount of PGE2 produced by endogenous enzymes. This suggests that the LY294002 is not acting on the endogenous COX1. Fig. 5*B* shows that if the arachidonic acid is provided after the production of LPS-induced COX2 (24 h), there are still demonstrable increases in PGE2 in the LY294002-treated cells. If LY294002 was acting primarily on PLA2, the added arachidonic acid should have eliminated the increase in PGE2 in the LY294002-treated group. The fact that we still see LY294002-induced increases in PGE2 with surplus amounts of arachidonic acid suggests that PI3K inhibition is acting at some point other than the release of arachidonic acid from phospholipids.

PI3K Inhibition Increases the Production of LPS-induced COX2 Protein—For these studies, alveolar macrophages were treated for 24 h with LPS and LPS with LY294002, whole cell lysates were obtained, and COX2 protein was evaluated by Western analysis. The addition of LY294002 caused an ~3-fold increase in the amount of COX2 protein after 24 h of LPS (Fig. 6*A*). In addition, we evaluated the effect of LPS and PI3K inhibition on COX1 levels. Fig. 6*B* shows that COX1 levels remain the same in control, Ly294002, LPS, and LPS with LY294002-treated cells. These data, combined with the previous experiment showing no effect of LY294002 on PLA2 activity or on the production of PGE2 by constitutive COX1, suggest that the increase in LPS-induced PGE2 in LY294002-treated cells is dependent on an increase in the production of COX2 protein.

PI3K Inhibition Increases the Production of LPS-induced COX2 mRNA—Alveolar macrophages were cultured with LPS with and without LY294002 for 6 or 24 h. RNA was isolated, and COX2 mRNA levels were analyzed via real-time RT-PCR. Fig. 7 demonstrates that by 6 h after LPS treatment, LY294002 had increased the level of COX2 mRNA. By 24 h, the difference between LPS-treated cells and LPS with LY294002-treated cells had expanded significantly. The increase in COX2 mRNA and protein suggests that PI3K inhibition regulates LPS-induced COX2 production positively.

PI3K Inhibition Increases the Half-life of COX2 mRNA—COX2 mRNA stability is regulated at the 3′ untranslated region. The most abundant COX2 mRNA contains an exceptionally long untranslated region (2.5 kb) that contains

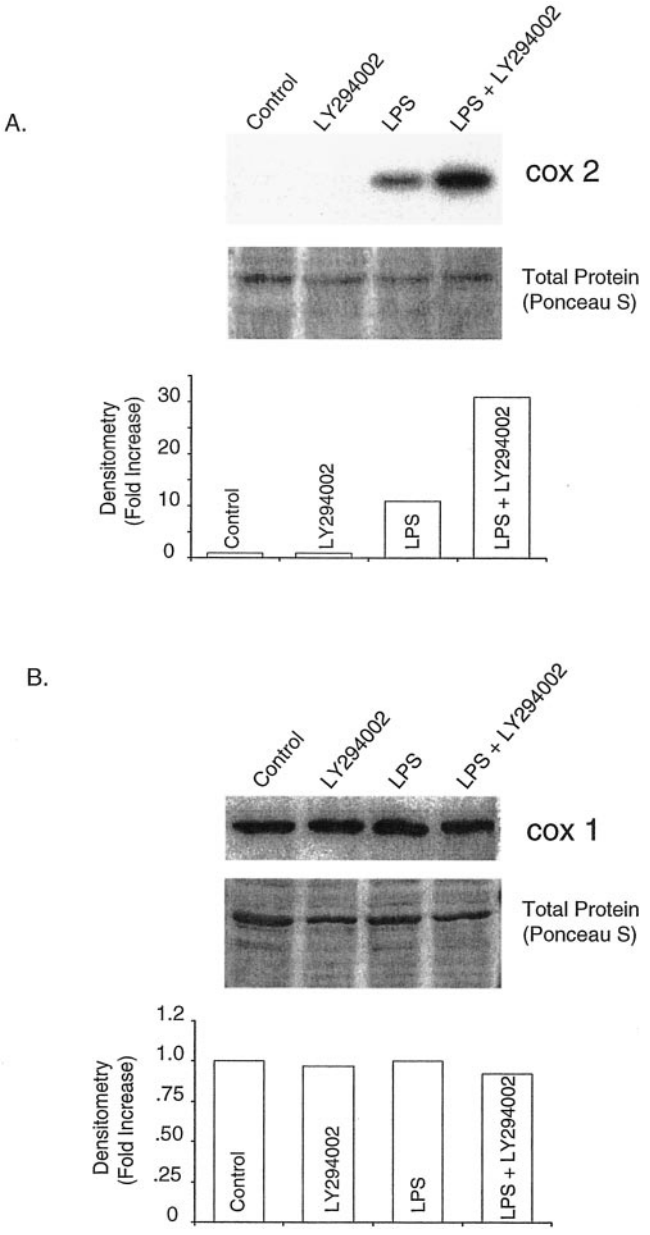

FIG. 6. **Inhibition of PI3K increases amounts of LPS-induced COX2, but not COX1, protein.** Alveolar macrophages were cultured for 24 h with and without LPS (100 ng/ml) and LY294002 (10 μm). Whole cell protein was obtained, and Western analysis was performed for COX2 (*A*) and COX1 (*B*). This is a representative of four separate experiments.

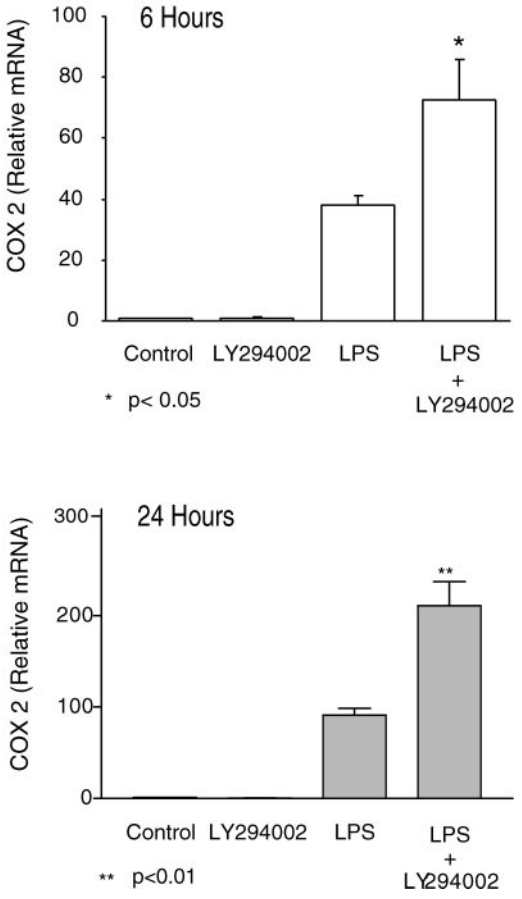

FIG. 7. **Inhibition of PI3K increases amounts of LPS-induced COX2 mRNA.** Alveolar macrophages were cultured for 6 or 24 h with and without LPS (100 ng/ml) and LY294002 (10 μm). mRNA was isolated, and COX2 message levels were determined by real-time PCR (see "Experimental Procedures"). The data represent three separate experiments. *, $p < 0.05$; **, $p < 0.01$.

multiple AUUUA repeats. Interactions at this region modulate the half-life of COX2 mRNA (27, 30, 31). For these studies, cells were cultured for 24 h with and without LPS and LY294002. At 24 h, actinomycin D was added and RNA harvested at 1, 2, and 3 h. Fig. 8*A* demonstrates that PI3K inhibition prolongs the half-life of LPS-induced COX2 mRNA. LPS-induced COX2 mRNA has a half-life of ~2 h. When PI3K activity is suppressed, ~80% of the COX2 mRNA remains at 3 h, and the mRNA half-life was ~8 h (data not shown). These data suggest that one mechanism by which PI3K inhibition increases LPS-induced PGE2 production is by extending the half-life of COX2 mRNA. Several studies have identified p38 as an upstream regulator of COX2 message stability (27, 30, 31, 41). To evaluate the role of p38 in the PI3K inhibition-induced COX2 mRNA stability, we added a p38 inhibitor 1 h before the addition of actinomycin D. The addition of a p38 inhibitor (SB202190) (42) to the PI3K-inhibited LPS sample decreased the mRNA stability almost to the levels of LPS alone (Fig. 8*B*). The p38 inhibitor was not added until 1 h before the actinomycin D, because we wanted to focus on mRNA stability and not p38-driven transcriptional effects. These data suggest that an increase in p38 activity is necessary for much (though not all) of the increased COX2 mRNA stability seen with PI3K inhibition.

PI3K Inhibition Increases Activation of p38 MAP Kinase—Because of the decrease in mRNA stability with p38 inhibition, we next evaluated the effect of PI3K inhibition on p38 activity. Cells were cultured with LPS, with and without LY294002, for 6 h, whole cell lysates were obtained, and activity of the p38 pathway was assessed. p38 is activated by the dual specificity kinase MKK6. p38 phosphorylates and activates the downstream kinase MAPKAP2. We used phosphorylation-specific antibodies that are linked to activating phosphorylations in MKK6, p38, and MAPKAP2. Fig. 9*A* demonstrates that inhibition of the PI3K pathway increases activation of MKK6, p38, and MAPKAP2. This occurs in both the presence and absence of LPS, suggesting that one role of the constitutive PI3K activity we find in alveolar macrophages is to dampen p38 activity. To determine whether this was a transient event or whether PI3K inhibition induced a prolonged activation of p38, we evaluated p38 activity over the course of several hours. Fig. 9*B* demonstrates a prolonged activation of p38 following PI3K inhibition. These data suggest that the increase in COX2 mRNA could result from an increase in mRNA stability generated by p38 activation. Activation of p38 alone was not suffi-

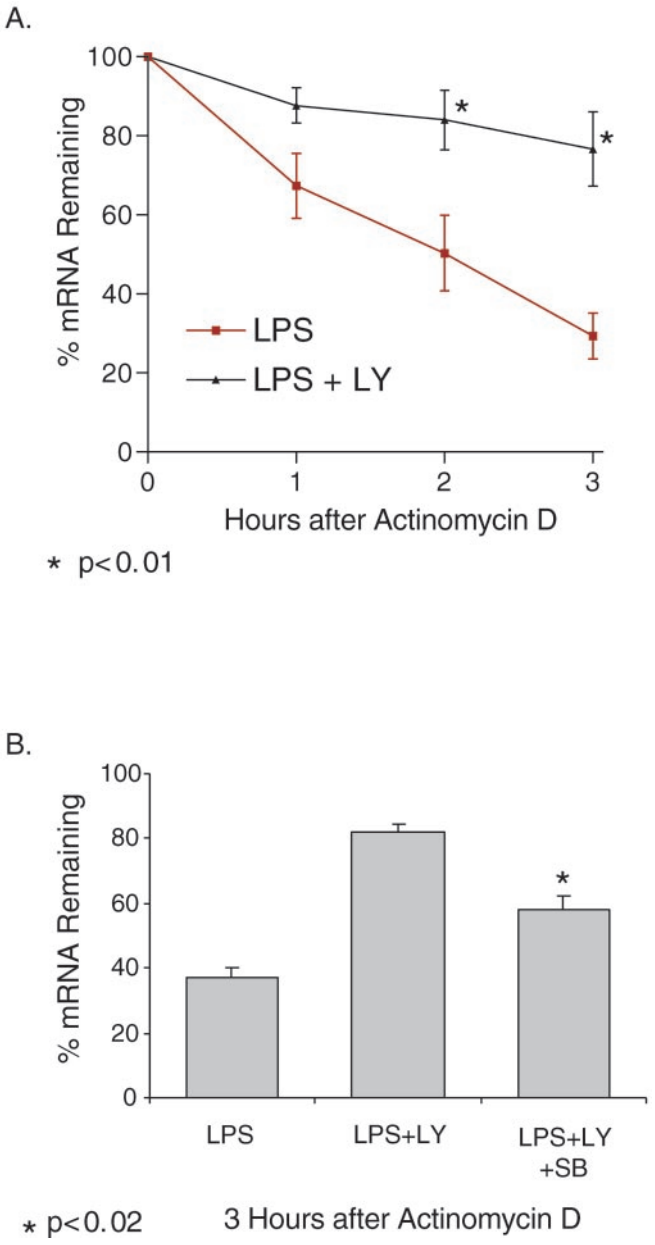

FIG. 8. **Inhibition of PI3K increases COX2 mRNA stability.** *A*, alveolar macrophages were cultured for an initial 24 h with and without LPS (100 ng/ml) and LY294002 (10 μm). At 24 h actinomycin D was added to the cultures, and mRNA was harvested after an additional 1, 2, or 3 h. COX2 mRNA levels were evaluated with real-time PCR. *B*, alveolar macrophages were cultured as in *A* except for the addition of a group with LPS (100 ng/ml) + Ly294002 (10 μm) and + SB202190 (10 μm). The LY294002 and LPS were added at the beginning of the experiment. The SB202190 was added at 23 h. The groups were analyzed for COX2 mRNA at 24 h and then again after 3 h for actinomycin D. The *graph* depicts the % mRNA remaining after 3 h of actinomycin D. The percent remaining is calculated as follows for both *A* and *B*: amount of mRNA after actinomycin D/amount of mRNA at 24 h $\times$ 100. The data represent three separate experiments for both *A* and *B*. *A*, *, $p < 0.01$ (comparison between LPS alone and LPS+LY); *B*, *, $p < 0.02$ (comparison between LPS+LY and LPS+LY+SB). There is no significant difference between LPS alone and LPS+LY+SB.

cient to induce COX2 and PGE2 (see Fig. 1 and Fig. 6). Therefore, LPS activates other signals that are involved in COX2 production.

Inhibition of p38 Decreases LPS-induced COX2 and PGE2—To confirm a role for p38 in the production of COX2 and PGE2, we used the p38-specific inhibitor, SB202190. The addition of SB202190 to LPS-treated alveolar macrophages resulted in a decrease in COX2 protein and PGE2 production (Fig. 10). These data demonstrate a direct link between p38 activity and PGE2 production.

PI3K Activity and p38 Activity Are Correlated Inversely in LPS-treated Alveolar Macrophages—To compare LPS-induced activation of both Akt and p38 we evaluated both events in the same sets of cells. Fig. 11 shows that LPS induces an early activation of p38 (1 min) that is gone by 5 min, the point when Akt activation by LPS appears. At later time points (6–12 h), Akt activity goes down, and p38 activity increases. The line graph shows that the early activation of PI3K parallels a block in p38 activation. These data support the inhibitor data demonstrating that PI3K inhibition increases p38 activity.

DISCUSSION

We have shown previously that LPS induces production of PGE2 in human alveolar macrophages by increasing amounts of COX2 protein and mRNA (1, 5, 6). We have also shown that LPS increases the activity of PI3K and its downstream kinase, Akt (13, 14). In this study, we show that PI3K activity regulates expression of COX2 and release of PGE2 negatively. A significant effect of active PI3K is to decrease stability of COX2 mRNA. We further showed that the PI3K pathway suppressed p38 MAPK activity, and active p38 regulates stability of the COX2 mRNA positively. Of interest, we also found that human alveolar macrophages have high constitutive PI3K activity, as well as LPS-induced PI3K activity. The expression of COX2 did not increase after LPS stimulation until there was a decrease in PI3K activity to below baseline level. This occurred at late time points after LPS stimulation. These studies suggest that PI3K activity must decrease below a threshold level to permit expression of COX2.

Fig. 12 shows the role we feel PI3K plays in COX2 expression. The figure shows formation of the well described Toll-like receptor (TLR 4) signaling pathway after macrophage LPS exposure (43–45). Downstream of this complex, signaling intermediates activate NFκB, all three MAP kinases (this diagram focuses on p38 only), and PI3K. The exact mechanism of PI3K activation after LPS is not known at this time, but it is known that there are two major pathways downstream of TLR 4, MyD88-dependent and MyD88-independent (46). Future studies should determine the exact upstream activators of PI3K. PI3K, via Akt, is known to be a positive regulator of NFκB activity, suggesting the need for some PI3K activity in COX2 transcription (47–49). Our studies, however, focus on the negative regulation of p38 by PI3K and its consequence (decreased COX2 mRNA stability). The end effect of PI3K activity in LPS-treated alveolar macrophages is the decreased production of PGE2.

This study doesn't exclude an effect on COX2 transcription by PI3K inhibition. In fact, we have shown that an active p38 is necessary for expression of both NFκB and AP-1 luciferase activity after LPS (NFκB) or phorbol myristate acetate (AP-1) (28, 50). This effect of p38 is mediated by phosphorylation of TATA-binding protein, which permits this component of the basal transcription complex to bind to the TATA box and interact physically with NFκB and AP-1 proteins. However, we believe the major regulatory effect of PI3K on COX2 is at the message stability level. Mestre *et al.* (51) have shown in a recent study that there is significant redundancy in the pathways that lead to COX2 transcription in monocyte/macrophages. They found that mutations in the NFκB, NFIL-6, or CRE sites alone did not change COX2 promoter activity. They also showed that dominant negative MAP kinases (ERK, p38, or JNK) did not decrease COX2 promoter activity.

Several previous studies have suggested a role for p38 in the stabilization of some mRNAs. A study by Guan *et al.* (52) in

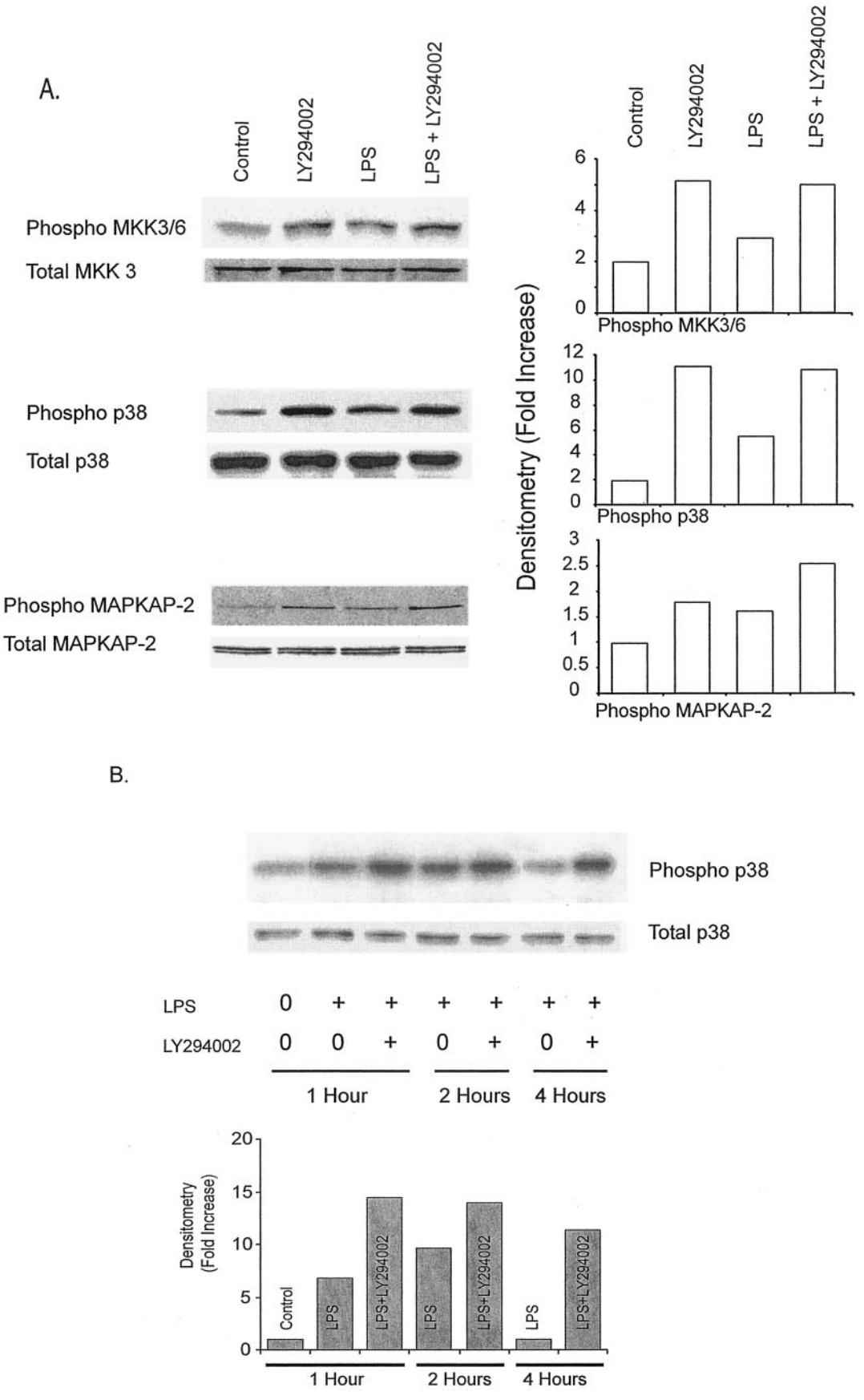

FIG. 9. **Inhibition of PI3K increases MKK3/6, p38, and MAPKAP2 activity.** *A*, alveolar macrophages were cultured for 6 h with and without LPS (100 ng/ml) and LY294002 (10 μm). Whole cell protein was obtained, and Western analysis was performed for activating phosphorylations in MKK3/6 (serine 189/207), p38 (threonine 180/tyrosine 182), and MAPKAP2 (threonine 334). This is representative of three separate experiments. *B*, alveolar macrophages were cultured for 1–4 h with and without LPS (100 ng/ml) and LY294002 (10 μm). Whole cell protein was obtained, and Western analysis was performed for activated p38 (threonine 180/tyrosine 182). This is a representative of three separate experiments.

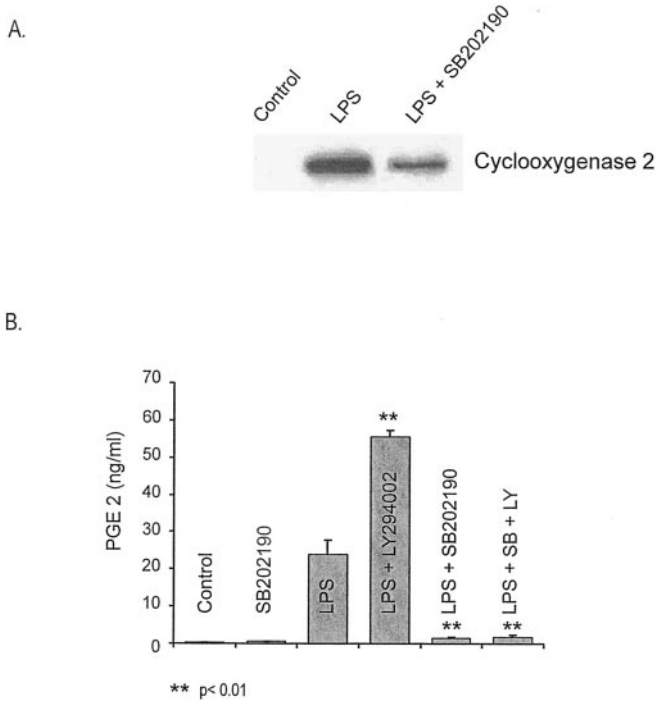

FIG. 10. **Inhibition of p38 decreases LPS-induced COX2 protein and PGE2.** *A*, alveolar macrophages were cultured for 24 h with and without LPS (100 ng/ml) and SB202190 (10 μm). Whole cell protein was obtained, and Western analysis was performed for COX2 protein. *B*, alveolar macrophages were cultured for 24 h with and without LPS (100 ng/ml) and SB202190 (10 μm). Supernatants were harvested, and PGE2 levels were determined by ELISA. The data represent three separate experiments. **, $p < 0.01$.

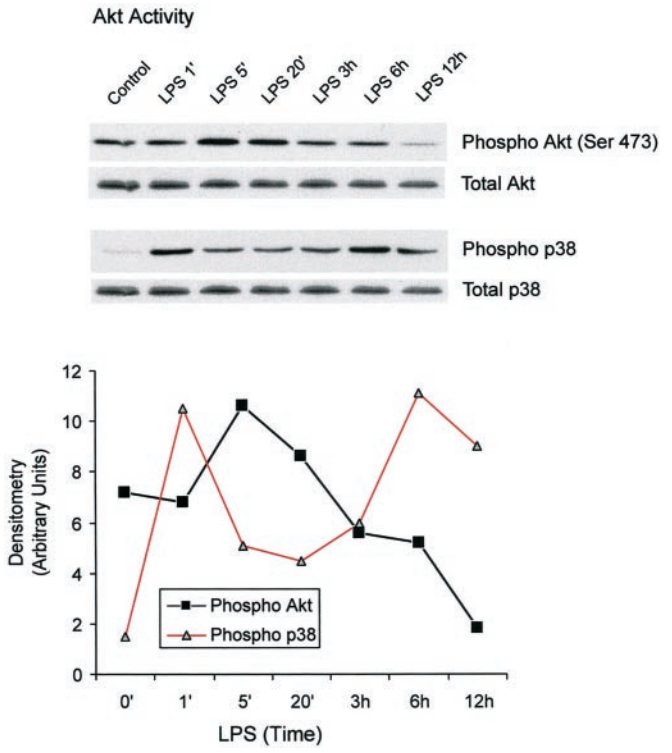

FIG. 11. **LPS-induced Akt activity is correlated inversely with LPS-induced p38 activity.** Alveolar macrophages were cultured with LPS (100 ng/ml) for various times (1 min to 12 h). Whole cell proteins were obtained, and Western analysis was performed for phosphorylated Akt (serine 473) and p38 (threonine 180/tyrosine 182). This is a representative of three separate experiments.

1998 links p38 activation to production of COX2. They showed that a dominant negative p38 upstream kinase would reduce expression of COX2. This study was followed in 2000 by a group of studies showing that p38 activity had a positive effect on COX2 mRNA stability (27, 31). The study by Clark and colleagues (27) used a tetracycline-regulated reporter system to investigate the regulation of COX2 mRNA stability. They found that a chimeric transcript with the COX2 3′ region was stabilized by a constitutively active MKK6, as well as an active MAPKAP2. They localized the p38 effect to the first 123 nucleotides 3′ to the stop codon (27). This same group later showed that dexamethasone inhibition of COX2 was because of a decrease in COX2 mRNA stability subsequent to inhibition of p38 (30). These studies on regulation of COX2 mRNA are consistent with our observation that PI3K inhibition increased p38 activity and stability of COX2 mRNA. p38 activation alone is not enough to induce COX2 production. In our cells, PI3K inhibition without LPS induced an increase in p38 activity but did not increase either COX2 or PGE2. This suggests that alternative pathways are activated by LPS (ERK, JNK, NFκB) that are necessary for transcriptional activation of the COX2 gene.

A possible negative role for PI3K in MAP kinase signaling has been described. A study by Park *et al.* (21) shows that Akt (downstream of PI3K) can inhibit SEK and JNK. Gratton *et al.* (53) have shown, in bovine aortic endothelial cells, that Akt phosphorylates and inactivates MEKK3 leading to a down-regulation of MKK3/6 and p38 activity. In Gratton's study, vascular endothelial growth factor-induced PI3K activity inhibits p38, protecting the cells from apoptosis. These studies, combined with our data, suggest that Akt may decrease activation of the stress kinases.

PI3K negative regulation of COX2 production is a possible explanation for the observation that IL-10 decreases COX2 (54). IL-10, like IL-13, has been shown to activate PI3K (55, 56), and in a separate study, IL-10 has also been shown to decrease p38 activity (57). This, in addition to our *in vitro* macrophage data, would suggest that modulation of PI3K activity levels may play an *in vivo* role in regulation of PGE2 production.

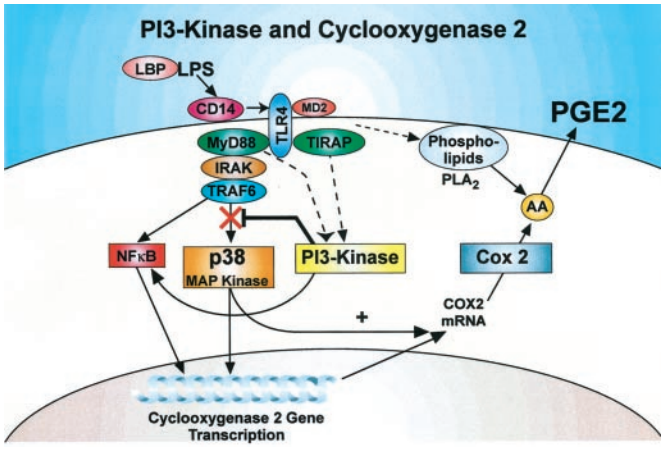

FIG. 12. **PI3K activity negatively regulates COX2 mRNA stability via inhibition of p38.** This is a diagram demonstrating the role of PI3K in LPS-induced COX2 and PGE2 production. Our data show that PI3K regulates p38 activity negatively leading to decreased mRNA stability, less COX2 production, and decreased PGE2 release. *AA*, arachidonic acid; *IRAK*, interleukin 1 receptor-associated kinase; *LBP*, lipopolysaccharide-binding protein; *MyD88*, myeloid differentiation factor 88; *TIRAP*, Toll-IL-1R-like homology domain-containing adaptor protein; *TLR4*, Toll-like receptor 4; *TRAF6*, tumor necrosis factor receptor-associated factor 6.

A few studies have evaluated regulation of COX2 by PI3K. Three of these studies described outcomes opposite of ours. Tang *et al.* (58) found that a dominant negative Akt suppresses

UV-induced COX2 in keratinocytes. In the Tang study, PI3K inhibition increased COX2 expression. A study by Sheng *et al.* (59) looked at intestinal epithelial cells and showed that a PI3K inhibitor blocked COX2 production by activated K-Ras. Finally, a study in colon carcinoma cells found that active Akt increased COX2 expression (60). The difference in findings between these studies and ours could be because of cell specificity (none of these cells are immune cells) or different stimuli. The only study that has shown PI3K inhibition increasing COX2 is a study by Weaver *et al.* (61). Using colonic epithelial cell lines, they showed that the inhibitor wortmannin increased COX2 protein, and the inhibitor LY294002 increased COX2 mRNA. Their data were limited to these observations. Our study utilizing human alveolar macrophages and a sepsis-relevant stimulus (LPS) is the first to show negative regulation of COX2 by PI3K activity in a primary human cell. In addition, it is the first study to show a link between PI3K and destabilization of COX2 mRNA.

In summary, the novel findings of this study include constitutive PI3K activity in primary macrophages (alveolar), PI3K-dependent suppression of p38 activity and COX2 mRNA stability, and increased LPS-induced COX2 and PGE2 production with PI3K inhibition. These observations suggest that there is both constitutive and inducible early negative regulation of COX2 activity. The late rise in COX2 activity coincides with an increase in p38 activity and a decline in PI3K activity.

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